Effect of heterologous and homologous seminal plasma on stallion sperm quality

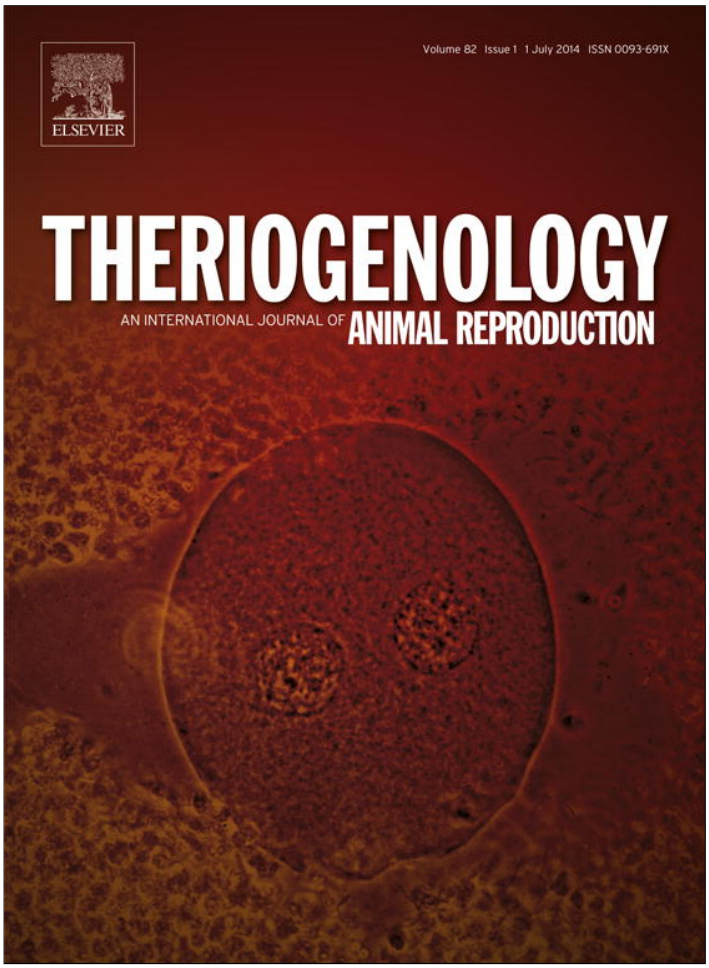
Morrell, Jane; Georgakas, Athanasios; Lundeheim, Nils; Nash, Deborah Mary; Davies-Morel, Mina Clare Gwynne; Johannison, A.

Published in:
Theriogenology

DOI:
10.1016/j.theriogenology.2014.03.020

Publication date:
2014

Citation for published version (APA):
Morrell, J., Georgakas, A., Lundeheim, N., Nash, D. M., Davies-Morel, M. C. G., & Johannison, A. (2014). Effect of heterologous and homologous seminal plasma on stallion sperm quality. *Theriogenology*, *82*(1), 176-183. https://doi.org/10.1016/j.theriogenology.2014.03.020



Theriogenology 82 (2014) 176–183

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Effect of heterologous and homologous seminal plasma on stallion sperm quality

J.M. Morrell [a,*], A. Georgakas [a,b], N. Lundeheim [c], D. Nash [b], M.C.G. Davies Morel [b], A. Johannisson [d]

[a] Department of Clinical Sciences, Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden
[b] Department of Biological, Environmental and Rural Sciences, Aberystwyth University, Wales, UK
[c] Department of Animal Breeding and Genetics, SLU, Uppsala, Sweden
[d] Department of Anatomy, Physiology and Biochemistry, SLU, Uppsala, Sweden

ARTICLE INFO

Article history:
Received 27 November 2013
Received in revised form 17 March 2014
Accepted 21 March 2014

Keywords:
Seminal plasma
Single layer centrifugation
Sperm kinematics
Sperm chromatin structure assay

ABSTRACT

Removing most of the seminal plasma (SP) from stallion semen has been shown to improve survival during cooled storage, yet adding small quantities of SP may improve pregnancy rates or cryosurvival. Furthermore, there is considerable controversy about whether the stallion's own SP or heterologous SP produces the best effect, possibly because of the variation between stallions in SP proteins or because some homologous SP remained in the sperm preparation. The SP is removed completely from stallion spermatozoa prepared by colloid centrifugation. Thus, the aim of the present study was (1) to investigate the effect of adding back SP to colloid centrifuged spermatozoa to determine its effect on spermatozoa; and (2) to investigate whether the stallion's own SP had a greater or lesser effect than heterologous SP. Conventional semen doses were sent from a stud overnight to the laboratory using standard transport conditions. Once at the laboratory, the semen samples were used for single layer centrifugation with Androcoll-E, and the resulting sperm preparations were treated with heterologous SP. Adding SP had a small but significant effect on sperm motility but no effect on the proportion of spermatozoa that had acrosome reacted. There were significant increases in hydrogen peroxide production and chromatin damage ($P < 0.001$). When homologous and heterologous SP were compared, considerable variation was observed between stallions, so that it was not possible to predict whether homologous or heterologous SP, or no SP, will produce the best motility for spermatozoa from any given stallion. Therefore, it is necessary to test different combinations of spermatozoa and SP to find the optimal effect on motility. The SP from most stallions increased reactive oxygen species and chromatin damage. In conclusion, the interaction between SP and spermatozoa depends on the origin of both SP and spermatozoa. If it is desirable to add SP to stallion sperm samples, it should be done directly before insemination rather than before storage, because of increased hydrogen peroxide production and sperm chromatin damage.

1. Introduction

There are conflicting reports about the benefits or otherwise of seminal plasma (SP) in many species [1], no more so than in the horse. Seminal plasma is the fluid part of semen, which functions to activate epididymal spermatozoa and transport them to the site of semen deposition, but it is known to exert an effect on the female reproductive tract as well and may regulate ovarian function [2]. Some components of SP are responsible for preventing sperm capacitation (decapacitation factors). Proteins found in SP are thought to modulate fertility [3,4] such as horse SP

* Corresponding author. Tel.: +4618671152; fax: +461867113545.
E-mail address: jane.morrell@slu.se (J.M. Morrell).

0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.03.020

proteins and other members of the family of cysteine-rich secretory proteins—CRISP proteins [5,6]. However, sperm samples that have had all SP removed by colloid centrifugation are fertile [7–9]. Semen plasma also has beneficial effects on spermatozoa, affecting sperm motility [10,11] in fresh sperm samples and after cryopreservation [12]. In mares susceptible to persistent breeding-induced endometritis, the presence of some SP in the artificial insemination (AI) dose may be necessary for optimum fertility [13], possibly acting via immune-regulatory molecules to activate and modulate immune responses [14].

In contrast to the benefits of SP, removal of most of the SP from stallion semen increases sperm survival during cold storage and reduces chromatin damage [15,16]. Cryopreservation protocols for stallion semen suggest removing more than 90% SP before adding the cryoextender [17] although Moore, et al. [18] did not observe a protective effect when various proportions of SP were included in cryopreserved semen. Mari, et al. [19] were unable to influence fertility in an AI trial by adding 5% SP to sperm doses immediately before deep intrauterine insemination of a small number of spermatozoa. Thus, the whole issue of the effects of SP is confusing.

The main problem with adding SP to ejaculated sperm samples to study its effects is that the spermatozoa may have already been activated by components of SP on ejaculation. Colloid centrifugation, such as single layer centrifugation (SLC), separates spermatozoa from SP and even removes SP proteins from the sperm surface [20]. Thus, known quantities of SP can be added to SLC-selected spermatozoa to enable the effects of SP to be measured. In previous studies, homologous SP was found to increase the motility and velocity of SLC-selected spermatozoa [10,21]. In a preliminary study comparing homologous and heterologous SP with a limited number of ejaculates, there was considerable variation between stallions as to whether homologous or heterologous SP produced the most effect. The aim of the present study, therefore, was (1) to investigate the effect of adding back SP to colloid centrifuged spermatozoa to determine its effect on spermatozoa; and (2) to investigate whether the stallion's own SP had a greater or lesser effect than heterologous SP.

2. Materials and methods

Semen collection was carried out according to standard veterinary and husbandry procedures. The experimental protocol was reviewed and approved by the Ethical Committee for Experimentation with Animals, Uppsala, Sweden (C345/9) before the start of the experiment.

2.1. Preparation of SP

Semen was collected from 10 warmblood stallions of known fertility, 4- to 15-years old, kept at a commercial stud in Sweden (Flyinge AB) during the nonbreeding season. Semen was collected up to three times per week for freezing, by allowing the stallions to mount a phantom and ejaculate into an artificial vagina (Colorado or Missouri, depending on the stallion). Gel was removed with an inline filter. Aliquots (10 mL) of semen were immediately centrifuged at 500× *g* for 10 minutes to pellet the spermatozoa and collect the SP supernatant. After checking the supernatant microscopically for the presence of spermatozoa, sperm-free SP aliquots were frozen individually until required. The seasonal pregnancy rates of these donor stallions after AI with cooled semen during the subsequent breeding season varied from 67% to 86%.

2.2. Semen collection

Semen was collected from 17 warmblood stallions of known fertility, 4- to 15-years old, kept at a commercial stud in Sweden (Flyinge AB) during the breeding season. Semen was collected as described previously. Sperm concentration was measured using a SpermaCue photometer (Minitüb GmbH, Tiefenbach, Germany), and motility was assessed subjectively. The ejaculate was extended in INRA96 (IMV Technologies, L'Aigle, France) to provide approximately 1 billion motile spermatozoa per dose (a standard cooled semen dose in Sweden). The doses were placed in insulated boxes with a cold pack for overnight transport, according to standard practice. The temperature inside the insulated box was approximately 6 °C.

2.3. Single layer centrifugation

On arrival at the laboratory at the Swedish University for Agricultural Sciences (approximately 24 hours after semen collection), the samples were allowed to equilibrate to room temperature before performing SLC. Aliquots (15 mL) were used for SLC [22]. Briefly, after equilibrating the semen and colloid to room temperature (approximately 22 °C) for 30 minutes, the extended semen from the commercial AI dose was carefully layered on top of Androcoll-E-Large (15 mL) in 50-mL Falcon tubes. After centrifugation at 300× *g* for 20 minutes, the resulting sperm pellet was aspirated into a clean tube containing 2 mL INRA96.

2.4. Sperm concentration

Sperm concentration in the SLC-selected samples was measured using a Nucleocounter SP-100 (ChemoMetic, Allerød, Denmark) as previously described [23]. INRA96 was added to a fixed number of spermatozoa (30×10^6) to provide a final volume of 0.5 mL after adding the SP (see experimental design).

2.5. Sperm evaluation

2.5.1. Computer-assisted sperm analysis

Motility assessment was made on both control and SLC-selected samples using a Sperm Vision (Minitüb, Tiefenbach, Germany), connected to an Olympus BX51 microscope (Olympus, Tokyo, Japan) with a heated stage (38 °C). Aliquots (6 μL) of the sperm samples were pipetted onto a warm glass slide, and an 18 × 18 mm cover slip was placed on top. Sperm motility of approximately 1000 spermatozoa in eight fields of view was analyzed using the Sperm Vision software program version 3.5 with previously established settings [24]. The cell identification area was set at 14 to 80 μm^2; spermatozoa having an average orientation change of head of less than 17° were defined as immotile, those

covering a straight line distance less than 6 μm or having a circular movement with a radius less than 35 μm and straight line distance less than 30 μm were defined as having local (i.e., nonprogressive) motility. The kinematics measured were total motility, progressive motility (PM), curvilinear velocity (VCL), straight line velocity (VSL), velocity of the average path (VAP), and linearity (LIN).

2.5.2. Sperm chromatin structure assay

Aliquots (50 μL) of the sperm samples were mixed with an equal volume of Tris–NaCl–EDTA buffer (0.15 M NaCl, 0.01 M Tris-HCl, 1 mM EDTA), pH 7.4, and were by snap-frozen in liquid nitrogen. They were subsequently stored at −80 °C for up to 3 months for evaluation using the sperm chromatin structure assay (SCSA). This method, which involved staining with acridine orange followed by flow cytometric analysis, has been reported previously [25]. The parameters quantified were the proportion of cells with single-stranded DNA (%DFI [%DNA fragmentation index]), the mean fluorescence of cells (mean_DFI), and the standard deviation (SD_DFI).

2.5.3. Acrosome reaction

The technique used to evaluate the proportion of spermatozoa that had undergone the acrosome reaction (AR) was that of Cheng, et al. [26] in which the binding of fluorescein isothiocynate–conjugated arachis hypogeal agglutinin to outer acrosomal membrane was measured. Briefly, 30 μL of the sperm sample was added to 270 μL of AR medium (30 mL INRA96, 10 mg Calcium chloride, 80 μL Calcium ionophore 5 mM), followed by 2.5 μL propidium iodide, 1 μL Hoechst 33342 (stock solution 5 mg/mL diluted 100 times), and 5 μL fluorescein isothiocynate–conjugated arachis hypogeal agglutinin (1 mg/mL). After incubation for 10 minutes, the stained cells were analyzed by flow cytometry using an LSR flow cytometer (Becton Dickinson, San José, CA, USA), equipped with standard optics. From each sample, a total of 10,000 events were collected and quantified as percentages.

2.5.4. Measurement of reactive oxygen species

The method used was a modification of Guthrie and Welch [27] reported previously for stallion spermatozoa [21]. Aliquots (500 μL) of extended semen were mixed with 2500 μL Cell-WASH (Becton Dickinson) and centrifuged at 400× *g* for 10 minutes. The supernatant was poured off, and 500 μL Cell-WASH was added to the sperm pellet. Two sets of samples were prepared for each sperm suspension as follows: 30 μL aliquots of the washed sperm suspension were added to 270 μL Cell-WASH and stained with Hoechst 33342 (Sigma, Stockholm, Sweden) (1.2 μM), hydroethidine (Molecular Probes Inc., Eugene, OR, USA) (1.2 μM), and dichlorodihydrofluorescein diacetate (Molecular Probes Inc.) (0.6 μM) to measure reactive oxygen species (ROS) production by flow cytometry. Menadione (Sigma-Aldrich, Stockholm, Sweden) (200 μM), a stimulant of ROS production, was added to one tube as a control that the spermatozoa were capable of producing ROS. After incubation at 37 °C for 30 minutes, the stained samples were analyzed by flow cytometry using the same instrument as described for the AR. The following subpopulations were identified and quantified as follows: living superoxide negative spermatozoa, living superoxide positive spermatozoa, dead superoxide positive spermatozoa, living hydrogen peroxide negative spermatozoa, living hydrogen peroxide positive spermatozoa, dead hydrogen peroxide negative spermatozoa, and dead hydrogen peroxide positive spermatozoa.

2.6. Statistics

Data analysis was performed using the statistical software package Statistical Analysis Systems software (version 9.2; SAS Institute Inc., Cary, NC, USA). Sperm quality parameters were examined using ANOVA (PROC MIXED). For experiment 1, the statistical model included the fixed effects of treatment (two groups: SLC and SLC + SP) stallion (n = 10), and the random effect of ejaculate nested within stallion. For experiment 2, the statistical model included the fixed effects of stallion (n = 9), treatment (10 classes; own SP [n = 1] and heterologous SP [n = 9]) and the interaction between stallion and treatment, and the random effect of ejaculate nested within stallion. Least squares means were calculated, and pair-wise tests of significance were performed.

2.7. Experimental design

2.7.1. Experiment 1

The effect of adding 5% heterologous SP on sperm motility, AR, ROS production, and chromatin damage was investigated using SLC-selected sperm samples from 10 stallions. The heterologous SP came from one individual. The analyses were performed immediately after adding the SP to the SLC-selected samples, except for chromatin damage where the sperm samples were stored for an additional 24 hours before removing aliquots for SCSA. A

Table 1
Effect of adding seminal plasma to SLC-selected stallion spermatozoa on sperm kinematics.

Treatment	TM	PM	LIN	VCL	VSL	VAP
SLC (24 h)	79 ± 1.6	53 ± 1.5*	0.42 ± 0.0**	123 ± 1.7***	52 ± 0.7***	67 ± 0.8***
SLC + SP (24 h)	80 ± 1.6	56 ± 1.5*	0.43 ± 0.0**	130 ± 1.7***	56 ± 0.7***	71 ± 0.8***
SLC (48 h)	74 ± 2.3	50 ± 1.9**	0.40 ± 0.0***	141 ± 2.3***	57 ± 0.8***	73 ± 1.7***
SLC + SP (48 h)	73 ± 2.3	45 ± 1.9**	0.39 ± 0.0***	156 ± 2.3***	61 ± 0.8***	81 ± 1.7***

24 hour and 48 hour refer to time after collection of semen. Thus, SLC and treatment with SP was done at 24 hours, with motility being evaluated again after a further 24-hour cold storage.
Values shown are LSMeans ± SE (n = 34).
Different letters within a column indicate significant differences between treatments for any given time as follows: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.
Abbreviations: LIN, linearity; LSMeans, least square means; NS, no significant difference between treatments; PM, progressive motility; SE, standard error; SLC, single layer centrifugation; SP, seminal plasma; TM, total motility; VAP, velocity of the average path; VCL, curvilinear velocity; VSL, straight line velocity.

Table 2
Effect of adding seminal plasma to SLC-selected stallion spermatozoa on the acrosome reaction.

Treatment	Live, non-AR (%)	Live, AR (%)	Dead, non-AR (%)	Dead, AR (%)
SLC	69 ± 2	0.4 ± 1.5	28 ± 2.4	0.3 ± 0.6
SLC + SP	67 ± 2	2.7 ± 1.5	28 ± 2.4	1.3 ± 0.6

The analysis was carried out immediately after adding SP. There were no significant differences between treatments.
Values shown are LSMeans ± SE (n = 34).
Abbreviations: AR, acrosome reacted; LSMeans, least square means; NS, no significant difference between treatments; SE, standard error; SLC, single layer centrifugation; SP, seminal plasma.

second motility measurement was also made at this time point, representing 48 hours postsemen collection and 24 hours post-SLC and SP treatment.

2.7.2. Experiment 2

The effect of adding 5% SP on sperm motility and chromatin integrity was assessed using ejaculates from nine stallions. In this case, the SP came from the same nine individuals, so that each stallion's sperm samples were tested with homologous SP and also heterologous SP from eight other individuals. Sperm motility was assessed immediately after adding the SP to an aliquot of SLC-selected sperm sample in each case. After 24 hours, sperm motility was assessed again, and an aliquot was taken for measurement of chromatin damage by SCSA.

3. Results

3.1. Experiment 1

Four samples were available from six stallions, three samples from two stallions, and two samples from two further stallions, giving a sample size of 34 altogether. Addition of SP to SLC samples resulted in an increase in PM ($P < 0.05$), LIN ($P < 0.01$), VCL ($P < 0.001$), VSL ($P < 0.001$) and VAP ($P < 0.001$) (Table 1). After a further 24-hour storage at 6 °C, PM had decreased in the SP-treated group compared with the SLC group ($P < 0.01$), although LIN, VCL, VSL, and VAP were all greater in the SP group ($P < 0.001$). Adding SP did not have any effect on the AR (Table 2).

For SCSA (Table 3), addition of SP significantly increased the proportion of spermatozoa with damaged chromatin ($P < 0.001$) and increased Mean_DFI ($P < 0.01$). There were also many significant differences with regard to ROS (Table 4): there was a trend for reduction in the proportion of living, superoxide negative spermatozoa after addition of SP ($P < 0.053$), and also a significant decrease in living superoxide positive and dead superoxide positive spermatozoa (both $P < 0.001$), a significant decrease in living, hydrogen peroxide negative spermatozoa, and significant increases in living and dead hydrogen peroxide positive (both $P < 0.001$), and dead hydrogen peroxide negative ($P < 0.05$) spermatozoa. Adding menadione significantly increased the proportions of all hydrogen peroxide positive spermatozoa and dead superoxide positive spermatozoa ($P < 0.001$) and decreased the proportions of ROS-negative spermatozoa and living superoxide positive spermatozoa ($P < 0.01$). The chromatin damage at this time point (24 hours after SLC and SP treatment) was related to ROS positivity measured on the previous day (immediately after SLC plus SP treatment) as follows (Fig. 1): living, superoxide positive and %DFI, $r = -0.44$ ($P < 0.001$); living peroxide negative and %DFI, $r = -0.31$($P < 0.01$); living peroxide positive and %DFI, $r = 0.25$ ($P < 0.01$); and dead peroxide positive, $r = 0.36$ ($P < 0.01$). Progressive motility and chromatin damage (Fig. 1) were also negatively associated, $r = -0.33$ ($P < 0.01$).

Table 3
Effect of adding seminal plasma to SLC-selected stallion spermatozoa on sperm chromatin, measured by the sperm chromatin structure assay.

Treatment	%DFI	Mean_DFI	SD_DFI
SLC	16 ± 1.1***	302 ± 2.2**	25 ± 0.4**
SLC + SP	28 ± 1.1***	314 ± 2.4**	26 ± 0.4**

Aliquots were frozen immediately after adding SP for subsequent analysis by SCSA.
Values shown are LSMeans ± SE (n = 34).
Different letters within a column indicate significant differences between treatments as follows: *** $P < 0.001$; ** $P < 0.01$.
Abbreviations: DFI, DNA fragmentation index; LSMeans, least square means; SCSA, sperm chromatin structure assay; SD, standard deviation; SE, standard error; SLC, single layer centrifugation; SP, seminal plasma.

3.2. Experiment 2

Adding SP to SLC-sperm samples did not have an effect on total motility or LIN, although there were differences between spermatozoa from different stallions. There was no significant interaction between spermatozoa and SP for both parameters. For progressive sperm motility (Table 5),

Table 4
Effect of adding seminal plasma to SLC-selected stallion spermatozoa on production of reactive oxygen species, (a) without menadione and (b) with menadione.

Treatment	Living, superoxide negative (%)	Living, superoxide positive (%)	Dead superoxide positive (%)	Living, hydrogen peroxide negative (%)	Living, hydrogen peroxide positive (%)	Dead, hydrogen peroxide negative (%)	Dead, hydrogen peroxide positive (%)
SLC	54 ± 1.5	9 ± 0.5**	37 ± 1.5***	63 ± 1.75***	0.4 ± 0.8***	36 ± 1.6	0.1 ± 0.7***
SLC + SP	52 ± 1.5	7 ± 0.5**	40 ± 1.5***	54 ± 1.75***	5.1 ± 0.8***	34 ± 1.6	5.4 ± 7***
SLC menadione	44 ± 2.6**	18 ± 2.4**	37 ± 1.4***	38 ± 3***	24 ± 2.8***	21 ± 1.8***	14 ± 2***
SLC + SP menadione	40 ± 2.6**	14 ± 2.4**	45 ± 1.4***	17 ± 3***	37 ± 2.8	13 ± 1.8***	31 ± 2***

Samples were analyzed immediately after adding SP.
Values shown are LSMeans ± SE (n = 34).
Different letters within a column indicate significant differences between treatments with or without menadione as follows: ** $P < 0.01$; *** $P < 0.001$.
Abbreviations: LSMeans, least square means; NS, no significant difference between treatments; SE, standard error; SLC, single layer centrifugation; SP, seminal plasma.

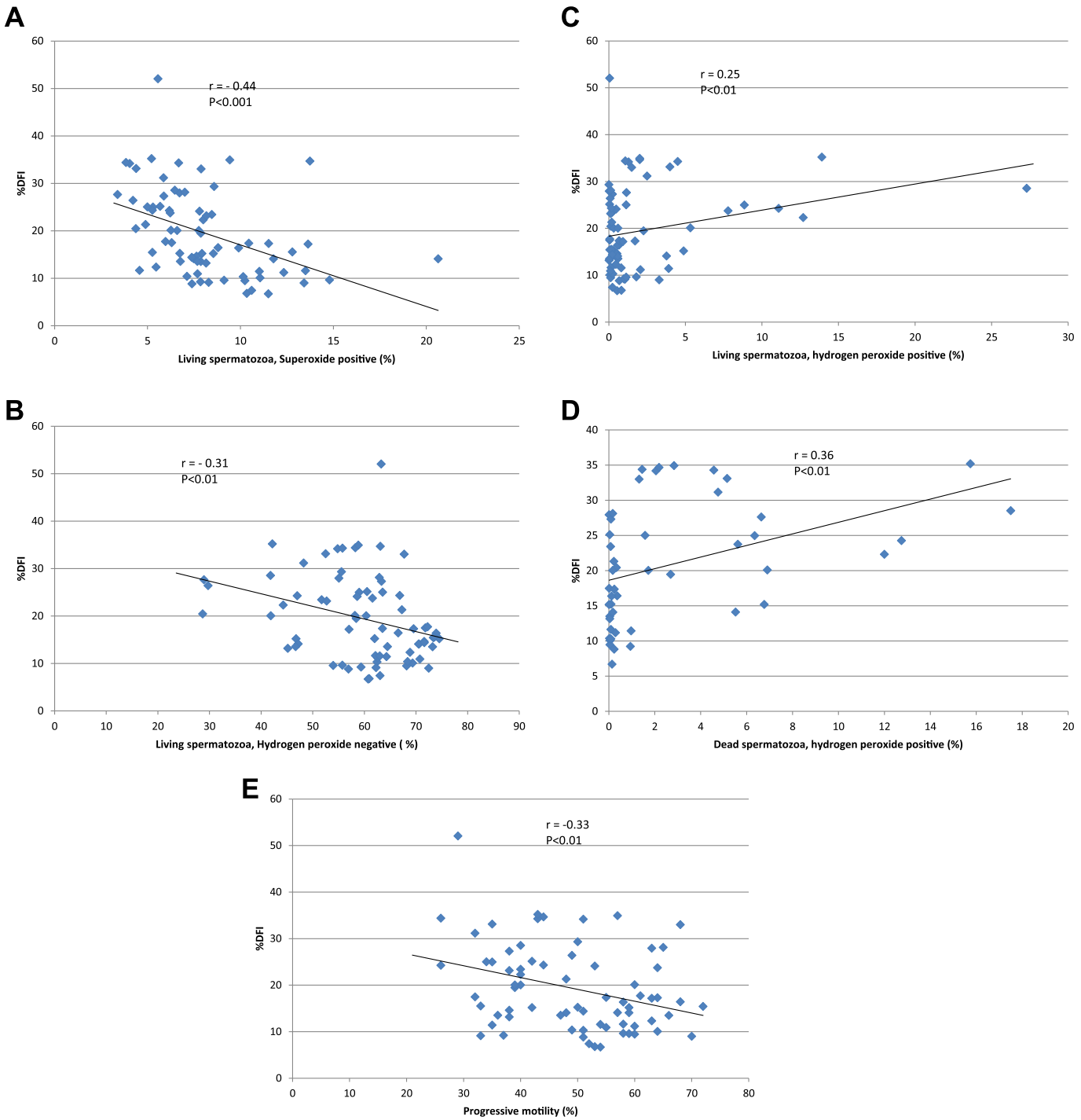


Fig. 1. Relationships between reactive oxygen species in SLC-selected sperm samples treated with seminal plasma and chromatin damage (%DFI) 24 hours later and between progressive motility and chromatin damage, both measured 24 hours after treatment of SLC-selected sperm samples with SP as follows (n = 73): (A) living, superoxide positive spermatozoa; (B) living, hydrogen peroxide negative spermatozoa; (C) living, hydrogen peroxide positive spermatozoa; (D) dead, hydrogen peroxide positive spermatozoa; and (E) progressive motility and chromatin damage. DFI, DNA fragmentation index; SLC, single layer centrifugation; SP, seminal plasma.

there was a significant difference between spermatozoa from different stallions ($P < 0.01$) and a trend for SP from different stallions to have an effect ($P < 0.067$). The interaction between spermatozoa and SP was not significant. For VCL, there were significant differences between spermatozoa from different stallions and different SP (both $P < 0.001$), but the interaction was not significant. For VSL and VAP, there were significant differences between spermatozoa from different stallions and different SP (both $P < 0.001$), and the interaction was also significant ($P < 0.01$). Two stallions (F and K) showed the best progressive motility for SLC samples that did not have SP added, whereas for three other stallions (A, E and I), the SLC samples showed the least progressive motility where no SP was added. The effect of adding SP on the progressive motility of spermatozoa from the other four stallions was variable, either increasing or decreasing progressive motility depending on the source of the SP.

Table 5
Effect of heterologous and homologous SP on motility (%) of SLC-selected spermatozoa.

Treatment	Sperm samples								
	A	B	C	D	E	F	I	J	K
SLC	55 ± 4	62 ± 4	63 ± 4	59 ± 4	32 ± 5	60 ± 4	60 ± 5	61 ± 5	68 ± 4
SLC + SPA	61 ± 4	60 ± 4	65 ± 4	56 ± 4	31 ± 5	58 ± 4	64 ± 5	63 ± 5	65 ± 4
SLC + SPB	62 ± 4	65 ± 4	63 ± 4	60 ± 4	41 ± 5	56 ± 4	59 ± 5	62 ± 5	66 ± 4
SLC + SPC	63 ± 4	58 ± 4	64 ± 4	61 ± 4	43 ± 5	56 ± 4	67 ± 5	62 ± 5	66 ± 4
SLC + SPD	60 ± 4	65 ± 4	64 ± 4	58 ± 4	37 ± 5	54 ± 4	65 ± 5	61 ± 5	66 ± 4
SLC + SPE	60 ± 4	65 ± 4	65 ± 4	63 ± 4	41 ± 5	55 ± 4	68 ± 5	63 ± 5	64 ± 4
SLC + SPF	60 ± 4	65 ± 4	66 ± 4	58 ± 4	39 ± 5	56 ± 4	65 ± 5	62 ± 5	67 ± 4
SLC + SPI	64 ± 4	67 ± 4	64 ± 4	59 ± 4	37 ± 5	54 ± 4	66 ± 5	63 ± 5	66 ± 4
SLC + SPJ	60 ± 4	64 ± 4	66 ± 4	59 ± 4	43 ± 5	57 ± 4	69 ± 5	61 ± 5	67 ± 4
SLC + SPK	60 ± 4	63 ± 4	65 ± 4	60 ± 4	44 ± 5	56 ± 4	64 ± 5	63 ± 5	65 ± 4
Summary									
SLC	55 ± 4	62 ± 4	63 ± 4	59 ± 4	32 ± 5	60 ± 4	60 ± 5	61 ± 5	68 ± 4
SLC + own SP	61 ± 4	65 ± 4	64 ± 4	58 ± 4	41 ± 5	56 ± 4	66 ± 5	61 ± 5	65 ± 5
SLC + foreign SP	61 ± 4	63 ± 4	65 ± 4	60 ± 4	39 ± 5	56 ± 4	65 ± 5	62 ± 5	66 ± 5

Differences between stallions overall $P < 0.001$; differences between SP overall trend toward significance ($P < 0.067$). Interaction between source of spermatozoa and source of SP $P < 0.001$. Foreign refers to the overall mean of all the heterologous SP treatments for sperm from each stallion. Samples were analyzed immediately after adding SP.
Values shown are LSMeans ± SE (n = 34).
Abbreviations: LSMeans, least square means; SE, standard error; SLC, single layer centrifugation; SP, seminal plasma; X, stallion identity.

The effect of adding SP on chromatin damage resulted in a greatly increased %DFI, except where SP from stallion I was used, which did not have any effect on the %DFI in any spermatozoa, regardless of source (Table 6). There were no differences between spermatozoa from different stallions but the differences between SP were significant ($P < 0.01$). The interaction was highly significant ($P < 0.001$). There were highly significant differences between spermatozoa from different stallions and also between SP for Mean_DFI and SD_DFI ($P < 0.001$); the interaction was significant for SD_DFI ($P < 0.01$) but not for Mean_DFI.

For eight stallions, their own SP produced as much chromatin damage as another stallion's SP, but SP from stallion I did not cause any damage to his own spermatozoa, whereas SP from other stallions did cause chromatin damage. The SLC-selected samples without added SP showed less chromatin damage (%DFI) than their uncentrifuged counterparts ($P < 0.001$). The variation among stallions was significant ($P < 0.001$).

4. Discussion

The aim of these experiments was to study the effect of adding SP to spermatozoa that had first been separated from their original SP by SLC. Furthermore, it was intended to investigate whether the origin of the SP influenced the result, i.e., whether the stallion's own SP had a greater or lesser effect than heterologous SP. The results showed that

Table 6
Effect of heterologous and homologous SP on chromatin damage (%).

Treatment	Sperm samples								
	A	B	C	D	E	F	I	J	K
Uncentrifuged	31 ± 4.3	27 ± 5	35 ± 4.3	36 ± 4.3	47 ± 6.1	37 ± 4.3	28 ± 6.1	33 ± 6.1	27 ± 4.3
SLC	13 ± 4.5	16 ± 5	22 ± 4.5	31 ± 4.6	34 ± 6.4	17 ± 4.5	15 ± 6.4	13 ± 6.4	15 ± 4.5
SLC + SPA	31 ± 4.5	27 ± 5	35 ± 4.5	35 ± 4.6	47 ± 6.4	37 ± 4.5	29 ± 6.4	33 ± 6.4	27 ± 4.5
SLC + SPB	37 ± 4.5	30 ± 5	38 ± 4.5	39 ± 4.6	55 ± 6.4	43 ± 4.5	34 ± 6.4	39 ± 6.4	32 ± 4.5
SLC + SPC	35 ± 4.5	28 ± 5	39 ± 4.5	37 ± 4.6	50 ± 6.4	39 ± 4.5	29 ± 6.4	35 ± 6.4	31 ± 4.5
SLC + SPD	36 ± 4.5	31 ± 5	41 ± 4.5	40 ± 4.6	52 ± 6.4	45 ± 4.5	33 ± 6.4	40 ± 6.4	31 ± 4.5
SLC + SPE	38 ± 4.5	29 ± 5	41 ± 4.5	38 ± 4.6	50 ± 6.4	42 ± 4.5	31 ± 6.4	40 ± 6.4	31 ± 4.5
SLC + SPF	35 ± 4.5	29 ± 5	37 ± 4.5	37 ± 4.6	49 ± 6.4	38 ± 4.5	30 ± 6.4	34 ± 6.4	28 ± 4.5
SLC + SPI	13 ± 4.5	16 ± 5	20 ± 4.5	26 ± 4.6	30 ± 6.4	18 ± 4.5	15 ± 6.4	13 ± 6.4	14 ± 4.5
SLC + SPJ	40 ± 4.5	31 ± 5	41 ± 4.5	41 ± 4.6	55 ± 6.4	48 ± 4.5	34 ± 6.4	41 ± 6.4	33 ± 4.5
SLC + SPK	35 ± 4.5	32 ± 5	38 ± 4.5	38 ± 4.6	51 ± 6.4	45 ± 4.5	33 ± 6.4	39 ± 6.4	31 ± 4.5
Summary									
Uncentrifuged	31 ± 4.3	27 ± 5	35 ± 4.3	36 ± 4.3	47 ± 6.1	37 ± 4.3	28 ± 6.1	33 ± 6.1	27 ± 4.3
SLC	13 ± 4.5	16 ± 5	22 ± 4.5	31 ± 4.6	34 ± 6.4	17 ± 4.5	15 ± 6.4	13 ± 6.4	15 ± 4.5
SLC + own SP	31 ± 4.5	30 ± 5	39 ± 4.5	40 ± 4.6	50 ± 6.4	38 ± 4.5	15 ± 6.4	41 ± 6.4	31 ± 4.5
SLC + foreign SP	34 ± 4.5	28 ± 5	36 ± 4.5	36 ± 4.6	49 ± 6.4	40 ± 4.5	32 ± 6.4	34 ± 6.4	28 ± 4.5

All SLC samples were significantly lower than uncentrifuged ($P < 0.001$). All samples treated with SP had significantly higher %DFI than SLC ($P < 0.001$), except for stallion I were there was no difference. Own seminal plasma had the same effect as foreign SP except for stallion I where own SP had no effect and foreign SP had a significant effect on %DFI ($P < 0.001$). Aliquots were frozen immediately after adding SP for subsequent analysis.
Values shown are LSMeans ± SE (n = 34).
Abbreviations: DFI, DNA fragmentation index; LSMeans, least square means; SE, standard error; SLC, single layer centrifugation; SP, seminal plasma.

adding SP in some cases had a small effect on sperm motility but had no effect on the proportion of spermatozoa that had acrosome reacted. There was, however, increased hydrogen peroxide positivity and increased chromatin damage.

These results are consistent with previous results from our laboratory where SLC-selected spermatozoa showed an increase in sperm motility when SP was added shortly after the SLC preparation was made, but no increase in motility was seen where the SLC-selected sperm samples were stored for 24 hours before adding the SP [21]. In the present study progressive motility was increased when SP was added, although the samples had been stored for 24 hours before performing the SLC and addition of SP. However, different motility analyzers were used for the two studies and also different stallions and different sperm samples (SLC performed on fresh samples vs. SLC on stored samples). In the previous experiment, the computer-assisted sperm analysis (CASA) instrument used at the stud was the Qualisperm Motility Analyzer that does not measure LIN, in contrast to the Sperm Vision used in the present study. Since the two instruments use different algorithms to measure kinematics, and do not report the same range of kinematics, it is not clear whether the difference in results is due to variation between stallions or between CASA instruments. The present results are also in contrast to those of de Andrade, et al. [28], who did not find any effect of adding SP to thawed spermatozoa on sperm motility or velocity, although LIN was reduced. In our experiment, LIN was increased by SP although this effect disappeared with subsequent storage for a further 24 hours. In addition, de Andrade, et al. [28] observed that beat cross frequency (BCF) and straightness (STR) were reduced in their experiment, whereas they were unaffected in the present study (BCF: 33.7 $\pm$ 2.4 vs. 33.7 $\pm$ 2.7; STR: 0.77 $\pm$ 0.02 vs. 0.78 $\pm$ 0.03; not significant). However, their methodology was different to ours, because their study did not remove all the SP from the spermatozoa to start with, they added SP to thawed sperm samples, they used a different CASA system and tested SP from only four stallions. The association between increased hydrogen peroxide positivity and increased chromatin damage and also the negative association between superoxide positivity and chromatin damage are consistent with our previous studies [22], in which we found that peroxide, but not superoxide, is associated with chromatin damage in stallion spermatozoa 24 hours later. Sperm motility in the present study, measured 24 hours after treatment, was also negatively correlated with %DFI, which is also in accordance with previous studies [22]. However, there was no association in the present study between hydrogen peroxide and decreased motility after storage, in contrast to previous studies [29] when exogenous hydrogen peroxide was added to sperm samples.

The observation that there is considerable variation between stallions in the effect of their SP on other stallions' spermatozoa is also consistent with other reports. In a previous study from our group, both homologous and heterologous SP caused a significant increase in chromatin damage during storage although homologous SP produced less damage than heterologous (Morrell and Johannisson, unpublished data). Kareskoski, et al. [11] also observed that the presence of SP during cooled storage caused DNA degradation but did not affect sperm motility. In the present study, the finding that one stallion (stallion I) had SP that did not negatively affect the chromatin integrity of any other stallion's sperm suggests that some stallions could serve as SP donors for others. However, one would need to test different combinations of SP and spermatozoa to identify such "universal SP donors".

Consideration should be given to the fact that the SP was prepared by centrifugation of semen, the sperm-free supernatant being subsequently frozen until used for the experiment. The effect of centrifugation on dead or dying spermatozoa could be to release intracellular contents or ROS into the SP, which then could have a deleterious effect on the sperm samples to which the SP was added. Thus, it is by no means clear that it is the SP itself that is having the deleterious effect on sperm quality but rather substances within the SP. However, it is still interesting that the SP from one stallion did not have a deleterious effect on sperm chromatin, since all SP samples were prepared and stored in the same way. This stallion did not differ from the others in terms of sperm quality in the original ejaculate from which the SP was prepared or in fertility.

Single Layer Centrifugation enables the effects of SP on spermatozoa to be studied because all the SP originally present in the semen is removed when the spermatozoa pass through the colloid, including the proteins coating the spermatozoa [20]. Thus, it is possible to compare the effects of heterologous versus homologous SP on spermatozoa. In cases where spermatozoa have been washed, i.e., by repeated centrifugation in semen extender or buffer, not all the SP is removed, especially the proteins coating the spermatozoa, which therefore makes it very difficult to quantify the effects of adding SP. Thus, differences between the results of the present study and other studies may be due to the presence or absence of all of the SP proteins. For example, a small cross-over study involving adding SP from sperm-rich and sperm-poor fractions concluded that any effect of SP on sperm motility or viability was lost after 24 hours [30,31].

4.1. Conclusions

In conclusion, the results presented here show that there is considerable variation between stallions in the effect of their SP on spermatozoa and that the interaction between SP and spermatozoa depends on the origin of both SP and spermatozoa. The SP from most stallions causes an increase in hydrogen peroxide production and chromatin damage. However, there is a suggestion that "universal SP donor" stallions may exist whose SP does not cause chromatin damage. Finally, the increased hydrogen peroxide production and sperm chromatin damage observed when SP is added before storage indicate that SP from most stallions should be added directly before insemination, if considered desirable, and not before storage.

Acknowledgments

We are grateful to Flyinge AB for supplying the semen samples used in this study. Funded by the Swedish Foundation for Equine Research. The sponsor was not involved in the study design; in the collection, analysis and

interpretation of data; in writing the report; or in the decision to submit the article for publication.

Author contributions: JM Morrell designed the study, provided the colloid, supervised the preparation of the semen samples by single layer centrifugation and the motility analysis, applied for the funding, drafted the manuscript; A Georgakis performed the technical work and prepared the database for statistical analysis; N Lundeheim did the statistical analysis; A Johannisson assisted with the design of the study and performed the flow cytometric analyses; all authors reviewed the manuscript.

Competing interests

JM Morrell is the inventor of Androcoll-E and has applied for a patent.

References

[1] Maxwell WMC, de Graaf SP, El-Hajj Ghaoui R, Evans G. Seminal plasma effects on sperm handling and female fertility. Soc Reprod Fertil Suppl 2007;64:13–38.

[2] Katila T. Sperm-uterine interactions: a review. Anim Reprod Sci 2001;68:267–72.

[3] Magdaleno L, Gasset M, Varea J, Schambony AM, Urbanke C, Raida M, et al. Biochemical and conformational characterisation of HSP-3, a stallion seminal plasma protein of the cysteine-rich secretory protein (CRISP) family. FEBS Lett 1997;420:179–85.

[4] Calvete JJ, Mann K, Schäfer W, Sanz L, Reinert M, Nessau S, et al. Amino acid sequence of HSP-1, a major protein of stallion seminal plasma: effect of glycosylation on its heparin- and gelatine-binding capabilities. Biochem J 1995;310:615–22.

[5] Cohen D, Busso D, Da Ros V, Ellerman D, Maldera J, Goldweic N, et al. Participation of cysteine-rich secretory proteins (CRISP) in mammalian sperm-egg interaction. Int J Dev Biol 2008;52:737–42.

[6] Töpfer-Petersen E, Ekhlasi-Hundrieser M, Kirchhoff C, Leeb T, Sieme H. The role of stallion seminal proteins in fertilisation. Anim Reprod Sci 2005;89:159–70.

[7] Varner DD, Love CC, Brinsko SP, Blanchard TL, David L. Hartman DL, Bliss SB, et al. Semen processing for the subfertile stallion. Journal of Equine Veterinary Science 2008;28:677–85.

[8] Morrell JM, Mari G, Kutvölgyi G, Meurling S, Iacono E, Mislei B, et al. Spermatozoa from stallion ejaculates processed by single layer centrifugation with Androcoll™-E are capable of fertilization after artificial insemination. Reprod Dom Anim 2011;46:642–5.

[9] Lindahl J, Dalin AM, Stuhtmann G, Morrell JM. Stallion spermatozoa selected by single layer centrifugation are capable of fertilization after storage for up to 96 h at 6°C prior to artificial insemination. Acta Vet. Scand 2012;54:40–4.

[10] Morrell JM, Rodriguez-Martinez H, Johannisson A. Single layer centrifugation of stallion spermatozoa improves sperm quality compared with sperm washing. Reprod Biomed Online 2010;21:429–36.

[11] Kareskoski M, Sankari S, Johannisson A, Kindahl H, Andersson M, Katila T. The association of the presence of seminal plasma and its components with sperm longevity in fractionated stallion ejaculates. Reprod Domest Anim 2011;46:1073–81.

[12] Kareskoski AM, Reilas T, Andersson M, Katila T. Motility and plasma membrane integrity of spermatozoa in fractionated stallion ejaculates after storage. Reprod Domest Anim 2006;41:33–8.

[13] Alghamdi AS, Foster DN, Troedsson MHT. Equine seminal plasma reduces sperm binding to polymorphonuclear neutrophils (PMNs) and improves the fertility of fresh semen inseminated into inflamed uteri. Reproduction 2004;127:543–600.

[14] Katila T. Post-mating inflammatory responses of the uterus. Reprod Domest Anim 2012;47(Suppl 5):31–41.

[15] Varner DD, Blanchard TL, Love CC, Garcia MC, Kenney RM. Effects of semen fractionation and dilution ratio on equine spermatozoal motility parameters. Theriogenology 1987;28:709–23.

[16] Love CC. The sperm chromatin assay: a review of clinical applications. Anim Reprod Sci 2005;89:39–45.

[17] Loomis PR, Graham JK. Commercial semen freezing: individual male variation in cryosurvival and the response of stallion sperm to customized freezing protocols. Anim Reprod Sci 2008;105:119–28.

[18] Moore AI, Squires EL, Graham JK. Effect of seminal plasma on the cryopreservation of equine spermatozoa. Theriogenology 2005;63:2372–81.

[19] Mari G, Castagnetti C, Rizzato G, Mislei B, Iacono E, Merlo B. Density gradient centrifugation of sperm from a subfertile stallion and effect of seminal plasma addition on fertility. Anim Reprod Sci 2011;126:96–100.

[20] Kruse R, Dutta PC, Morrell JM. Colloid centrifugation removes seminal plasma and cholesterol from boar spermatozoa. Reprod Fertil Dev 2011;23:858–65.

[21] Morrell JM, Pihl J, Dalin A-M, Johannisson A. Restoration of seminal plasma to stallion spermatozoa selected by colloid centrifugation increases sperm progressive motility but is detrimental to chromatin integrity. Theriogenology 2012;78:345–52.

[22] Morrell JM, Winblad C, Georgakas A, Stuhtmann G, Humblot P, Johannisson A. Reactive oxygen species in stallion semen can be affected by season and colloid centrifugation. Anim Reprod Sci 2013;140:62–9.

[23] Morrell JM, Johannisson A, Juntilla L, Rytty K, Bäckgren L, Dalin A-M, et al. Stallion sperm viability, as measured by the Nucleocounter SP-100 is affected by extender and enhanced by Single Layer Centrifugation. Vet Med Int. http://dx.doi.org/10.4061/2010/659862.

[24] Schäfer-Somi, Aurich C. Use of a new computer-assisted sperm analyzer for the assessment of motility and viability of dog spermatozoa and evaluation of four different semen extenders for predilution. Anim Reprod Sci 2007;102:1–13.

[25] Johannisson A, Morrell JM, Thoren J, Jönsson M, Dalin A-M, Rodriguez-Martinez H. Colloidal centrifugation with Androcoll-E prolongs stallion sperm motility, viability and chromatin integrity. Anim Reprod Sci 2009;116:119–28.

[26] Cheng FP, Fazeli A, Voorhout WF, Marks A, Bevers MM, Colenbrander B. Use of peanut agglutinin to assess the acrosomal status and the zona pellucida-induced acrosome reaction in stallion spermatozoa. J Androl 1996;17:674–82.

[27] Guthrie HD, Welch GR. Determination of intracellular reactive species and high mitochondrial membrane potential in Percoll-treated viable boar sperm using fluorescence-activated flow cytometry. J Anim Sci 2006;84:2089–100.

[28] de Andrade AFC, Zaffalon FG, Celeghini ECC, Nascimento J, Tarrago OFB, Martins SMMK, et al. Addition of seminal plasma to post-thawing equine semen: what is the effect on sperm cell viability? Reprod Domest Anim 2011;46:682–6.

[29] Baumber J, Vo A, Sabeur K, Ball B. Generation of reactive oxygen species by equine neutrophils and their effect on motility of equine spermatozoa. Theriogenology 2002;57:1025–33.

[30] Ackay E, Reilas T, Andersson M, Katila T. Effect of seminal plasma fractions on stallion sperm survival after cooled storage. J Vet Med A Physiol Pathol Clin Med 2006;53:481–5.

[31] Kareskoski AM, Katila T. Components of stallion seminal plasma and the effects of seminal plasma on sperm longevity. Anim Reprod Sci 2008;107:249–56.